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Brief report

Improved quantification of protein in vaccines containing aluminum hydroxide by simple modification of the Lowry method

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ABSTRACT

Aluminum (Al) components in vaccines are known to act as adsorbents that interfere with accurate protein quantification by the Lowry method. Therefore, certain modifications based on the characteristics and compositions of the vaccine are required for determination of protein contents.

We investigated the effects of an additional centrifugal separation and found that protein contents were overestimated by up to 238% without centrifugation through a collaborative study performed with hepatitis B vaccines containing Al. However, addition of a centrifugation step yielded protein concentrations that were similar to the actual values, with small coefficients of variation (CVs). Proficiency testing performed in 11 laboratories showed that four laboratories did not have satisfactory results for vaccines containing aluminum hydroxide, although all laboratories were proficient in protein analysis when samples did not contain aluminum hydroxide. Incomplete resuspension of aluminum hydroxide solution with alkaline copper solution was the major cause of insufficient proficiency in these laboratories.

1. Introduction

According to Korean regulations, U.S. Pharmacopoeia [1], and European Pharmacopoeia [2], the Lowry method is thought to be the most accurate method for determining the protein concentrations of licensed vaccines. However, various substances used for vaccine production, such as sucrose, Triton X-100, EDTA, Tris–HCl, lactose, and amino acid derivatives, may interfere with protein determinations [3–10]. Therefore, several modifications, including such as sodium deoxycholate (DOC) treatment, trichloroacetic acid (TCA) precipitation, or heat treatment, have been investigated for removal of interfering substances [13,14]; these modifications are included in the method referred to as the "conventional Lowry method." Unfortunately, these techniques are not effective for eliminating the interference of aluminum hydroxide [$Al(OH)_3$], a commonly used adsorbent in vaccine production. Moreover, while direct alhydrogel formulation immunoassays (DAFIAs) [11] or *O*-phthalaldehyde (OPA) assays [12] have been used for minimization of aluminum interference, these methods do not satisfy quality control standards due to insufficient sensitivity, poor reproducibility, and challenges with the preparation of antigens specific to the target proteins.

Prior to the initiation of this study, we reviewed the standard operating procedures (SOPs) of the Lowry method used by vaccine manufacturers and vaccine quality testing institutions. Several laboratories used only the conventional Lowry method to test vaccines containing $Al(OH)_3$, without the interference removal step. Therefore, we designed an Al-adjusted Lowry method for protein quantification of vaccines containing $Al(OH)_3$ by adding a centrifugation step to the conventional Lowry method. We then performed collaborative and proficiency studies and proposed a methodological adjustment for overcoming $Al(OH)_3$ interference.

2. Materials and methods

2.1. Reference standard for protein quantification

Pierce Bovine Serum Albumin (BSA) Standard (cat no. 23209; Thermo Fisher Scientific Inc.) was used as a reference standard.

Abbreviations: Al, aluminum; CV, coefficient of variation; DOC, sodium deoxycholate; TCA, trichloroacetic acid; $Al(OH)_3$, aluminum hydroxide; DAFIA, direct alhydrogel formulation immunoassay; OPA, *O*-phthalaldehyde; SOP, standard operative procedure; NIFDS, National Institute of Food and Drug Safety Evaluation; BSA, bovine serum albumin; IQR, interquartile range; ANOVA, analysis of variance.

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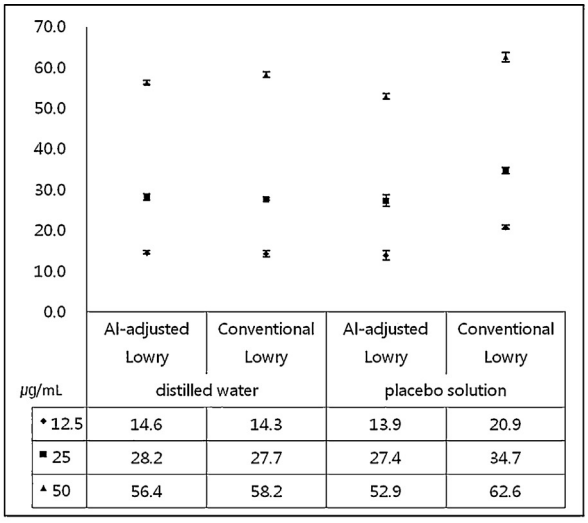
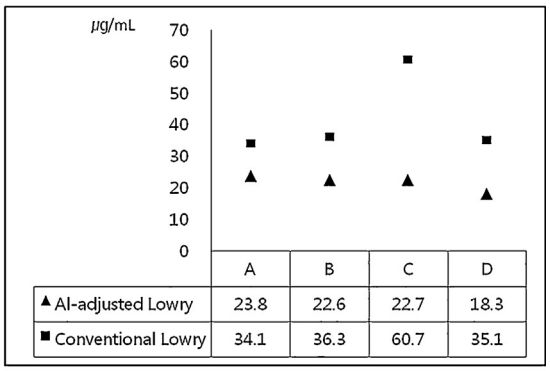
A



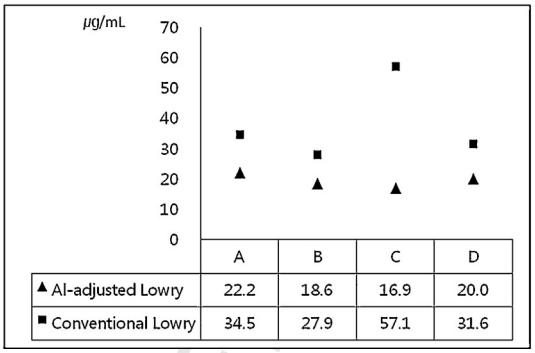
μg/mL	Al-adjusted Lowry (distilled water)	Conventional Lowry (distilled water)	Al-adjusted Lowry (placebo solution)	Conventional Lowry (placebo solution)
◆ 12.5	14.6	14.3	13.9	20.9
■ 25	28.2	27.7	27.4	34.7
▲ 50	56.4	58.2	52.9	62.6

B



	A	B	C	D
▲ Al-adjusted Lowry	23.8	22.6	22.7	18.3
■ Conventional Lowry	34.1	36.3	60.7	35.1

C



	A	B	C	D
▲ Al-adjusted Lowry	22.2	18.6	16.9	20.0
■ Conventional Lowry	34.5	27.9	57.1	31.6

Fig. 1. Verification of the accuracy of the Al-adjusted Lowry method. (A) Three BSA concentrations (12.5 [◆], 25 [■], and 50 [▲] μg/mL) were diluted with distilled water and placebo solution. Assays were performed independently on three different days. Each data point represents the mean ± standard deviation. (B) Three vaccine manufacturers and NCLR performed protein quantification using Euvax-B Inj. as a sample containing Al in a collaborative study. To assess the effects of application of a centrifugal separation step, two values from samples subjected to centrifugal separation (▲) or not (■) are presented. (C) Identical assays were performed using Hepavax-Gene TF Inj.

2.2. Placebo solution

Placebo solution containing buffers, isotonic agents, and $Al(OH)_3$, but not hepatitis B antigen, was obtained from a hepatitis B vaccine manufacturing company to confirm the interference of $Al(OH)_3$ on protein quantification.

2.3. Protein quantification by the Al-adjusted Lowry method

BSA was diluted serially to concentrations ranging from 5 to 100 μg/mL. Each standard solution and sample were mixed with 10% TCA and immersed in boiling water for 15 min. After cooling at room temperature and centrifugation at 3700 × *g* for 20 min, the precipitate was resuspended by adding 5% TCA solution followed by centrifugation at 3700 × *g* for 20 min, and the supernatant was then removed. The precipitate was resuspended by addition of alkaline copper solution. The diluted phenol reagent was then added, and the precipitate solution was incubated for 30 min at 37 °C.

For samples containing $Al(OH)_3$, an additional centrifugation step at 2000 × *g* for 5 min was included for separating interfering material. The supernatant was then collected, and the absorbance was measured at 750 nm.

2.4. Comparison of BSA quantification in distilled water and placebo solution

BSA was diluted with distilled water and placebo solution to 12.5, 25, or 50 μg/mL; thus, the test represented the target protein concentration of approximately 20 μg/mL, which is used for hepatitis B vaccine production. We performed the protein assay using conventional and Al-adjusted Lowry methods and compared the results.

2.5. Collaborative study

A collaborative study was performed using vaccines containing $Al(OH)_3$, Euvax-B Inj. (LG Life Sciences Ltd.) and Hepavax-Gene TF (Berna Biotech Korea Corp.), by the conventional and Al-adjusted Lowry methods. Three domestic vaccine manufacturers and the NIFDS participated, and all laboratories conducted separate assays based on a controlled protocol.

2.6. Proficiency testing

Eight vaccine manufacturers, two vaccine quality testing institutions, and the NIFDS participated using samples without Al (Korean

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